JOURNAL OF BACTERIOLOGY
Vol. 87, No. 5, pp. 1180–1191 May, 1964


# ULTRASTRUCTURE OF *TREPONEMA MICRODENTIUM* AND *BORRELIA VINCENTII*

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Received for publication 4 December 1963

## ABSTRACT

BLADEN, HOWARD A. (National Institute of Dental Health, Bethesda, Md.), AND EDWARD G. HAMPP. Ultrastructure of *Treponema microdentium* and *Borrelia vincentii*. J. Bacteriol. **87**:1180–1191.—A small oral *Treponema* (FM) and *Borrelia vincentii* (N9) were harvested after 3 to 7 days of incubation and either embedded in Vestopal W or negatively stained with phosphotungstate. The protoplasmic cylinders of both strains were identical except for size, and had a triple-structured cell wall as well as intracellular concentric laminations. Protoplasmic cylinders of both strains were enclosed in a cell envelope which appeared amorphous in negatively stained preparations, but which had a triple-structured wall when viewed in thin sections. The cell envelope of strain FM also acted as an envelope for the terminal filament; no filament envelope was evident in strain N9. Large structures which contained variable numbers of organisms and which were representative of spirochetal granules were observed. Protoplasmic cylinders contained within such granules frequently were devoid of cell envelopes. The axial filament consisted of several individual fibers which usually terminated in small end knobs. Occasionally, a fiber of the axial filament became a fiber of the terminal filament. Fibers of the terminal filament originated in end knobs similar to, but separate from, those to which the axial filament was attached. A periodicity of 60 A was occasionally observed in the terminal filament envelope of strain FM. A microperiodicity of approximately 20 A was also observed. The fibers of the terminal filament of strain N9 were composed of a large number of fibrils approximately 15 A wide. The periodicity and fibrillar structure of the terminal filament is discussed with reference to proposed models of bacterial flagella suggested by X-ray diffraction data.

Organisms of the genera *Treponema* and *Borrelia* present many morphological features which have been the subject of considerable controversy. Questions concerning the cell envelope, granules, and the disposition of the axial and terminal filaments, as well as motility, have been raised by various authors.

Noguchi (1928), using optical microscopy, concluded that a *Treponema* consisted of an axial filament and a layer of contractile protoplasm enclosed in a periplast. He believed that the axial filament was responsible for movement of the organism and that it differed from a true flagellum only in that it was intracellular and not extracellular. The first electron micrographs of spirochetes usually showed a cell envelope, an axial filament, and a protoplasmic cylinder, as well as a large number of fine threadlike appendages frequently interpreted as flagella or terminal filaments (Wile, Picard, and Kearney, 1942; Wile and Kearney, 1943; Mudd, Polevitzky, and Anderson, 1943; Hampp, Scott, and Wyckoff, 1948). Swain (1955), in a study of five species of spirochetes pathogenic for man, observed no true flagella. Listgarten, Loesche, and Socransky (1963) demonstrated the position of the axial filament with respect to the cell envelope and protoplasmic cylinder in thin sections of *T. microdentium*, but made no mention of flagella or terminal filaments.

With the development of newer methods of specimen preparation for electron microscopy, a better understanding of the ultrastructure of this intriguing group of microorganisms is made possible. In this paper, investigations on the ultrastructure of two strains of oral spirochetes are described.

## MATERIALS AND METHODS

The oral spirochetes employed in this study included a small oral *Treponema* (FM) and *B. vincentii* (N9) [Hampp et al., 1948].

The microorganisms were cultivated in veal heart broth which was essentially a modification of Huntoon's hormone agar (Fitzgerald and Hampp, 1952). At the time of use, an appropri-

ate quantity of the medium was heated in a boiling-water bath, cooled, and enriched with 10% canine ascitic fluid and 0.1% reduced glutathione. Both strains of spirochetes were grown for 3 to 7 days at 35 C in screw-cap tubes filled to capacity with the finished medium.

The spirochetes were harvested by centrifugation and either embedded for thin sectioning or negatively stained. The methods of fixation and embedding in Vestopal W were generally those described by Kellenberger, Ryter, and Séchaud (1958). In some cases, Epon was also used as an embedding medium. Sections were cut with an LKB microtome, stained with uranyl acetate, and examined in a Siemens Elmiskop I electron microscope at initial magnifications of up to 80,000×.

The negative staining technique was as follows: after centrifugation, the resultant pellet was resuspended in 0.85% NaCl solution, and a drop was placed on a Formvar-covered grid for 15 to 30 sec. This drop was removed and replaced with a drop of 2% phosphotungstic acid (adjusted to pH 5.0 with KOH) which was immediately removed.

## RESULTS

The two strains of spirochetes reported here were of the same general morphology, except for size, and consisted basically of a protoplasmic cylinder, a cell envelope, and associated filaments. Spirochetal granules which contained numerous protoplasmic cylinders were also observed frequently.

*Protoplasmic cylinder.* The cell wall of the protoplasmic cylinder was similar to that of many bacteria and appeared as two dense layers (20 to 25 A) separated by a less dense layer of approximately 30 A (Fig. 1, 2, and 3). A cytoplasmic membrane was not seen within the borders of the protoplasmic cylinder. Occasionally, concentric laminations occurred interior to the cell wall and were morphologically identical to it (Fig. 1). The repeated curvatures of strains FM and N9 were fairly regular, with strain N9 having an amplitude greater than that of FM. Frequently, cells were observed which did not have the characteristic spiral shape and which were almost straight. The axial filaments of these organisms often were broken and displaced (Fig. 4). The ends of the protoplasmic cylinder of strain FM were tapered and usually appeared as three bulbous areas in a row (Fig. 4). Strain N9, on the other hand, possessed slightly rounded ends (Fig. 5). Routinely, negatively stained cells had round-to-oval structures which appeared as though the cell wall of the protoplasmic cylinder had ruptured, laying open the internal portion of the cylinder (Fig. 4).

*Cell envelope.* In thin sections, a cell envelope immediately surrounding the individual protoplasmic cylinders was observed. In both strains, this structure appeared as two dense layers (20 to 30 A) separated by a less-dense layer (20 to 30 A), and was usually within 500 A of the protoplasmic cylinder (Fig. 1 and 2). No continuity was observed between the protoplasmic cylinder and the cell envelope. In negatively stained preparations, an amorphous layer covered the protoplasmic cylinder (Fig. 4, 5, 6, 8, 9, and 10). The contour of the amorphous layer was irregular, and, in strain N9, many projections resembling pseudopodia were seen (Fig. 5). These projections were usually rod-shaped and varied in length; many were constricted at their base. Occasionally, areas which contained similar configurations independent of the cell envelope were seen.

*Granules.* Spirochetal granules were frequently observed in thin-sectioned material of both strains FM and N9. They varied from 0.7 to 25 $\mu$ in diameter, and contained 2 to more than 50 protoplasmic cylinders. The limiting membrane was two dense layers of approximately 25 A separated by a less-dense layer (25 to 35 A). In many cases, the protoplasmic cylinders within a granule had cell envelopes (Fig. 2); in others, no cell envelopes were observed (Fig. 3). In the latter instance, axial filaments were not observed immediately adjacent to the cell wall (Fig. 2 and 3). No continuity between the cell envelope and the limiting membrane of the granule was evident.

*Axial and terminal filaments.* The axial filament was composed of one to six fibers approximately 200 A wide. They were usually parallel to each other (Fig. 6), and were contained in the space between the cell envelope and the protoplasmic cylinder (Fig. 2). The individual fibers of separate organisms ranged from 140 to 290 A, but those of a single organism were usually comparable in width. The space between the individual fibers usually appeared constant (Fig. 6), except at the ends of the protoplasmic cylinder where fibers occasionally were separated. Each

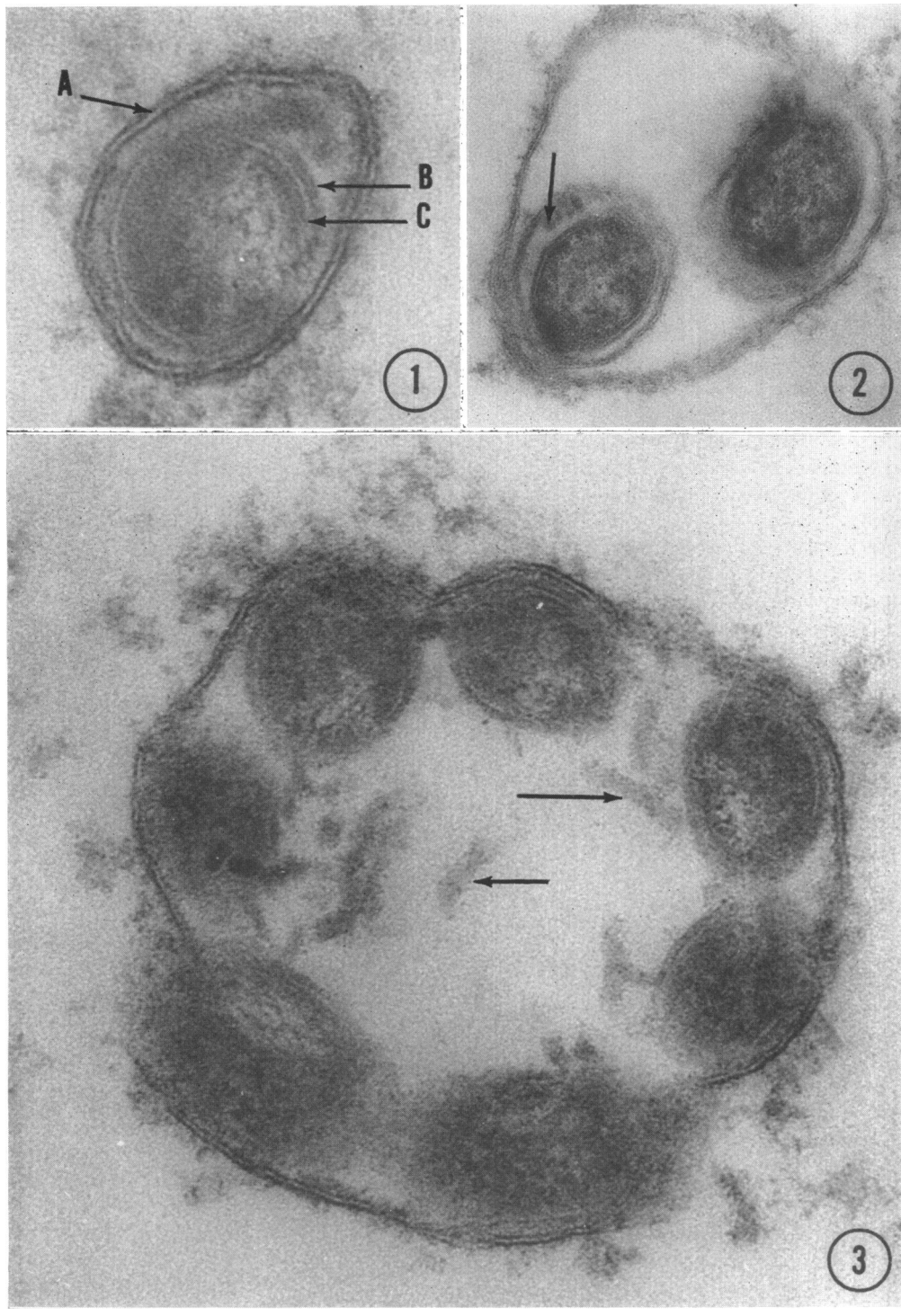


FIG. 1. *The triple layered cell envelope (A), as well as the cell wall (B) and concentric lamination (C) of the protoplasmic cylinder, are seen in a thin section of strain FM.* × *227,000.*

FIG. 2. *A small spirochetal granule of strain N9. The border of the granule, the cell envelope, and the cell wall of the protoplasmic cylinder are similar. Four axial filaments (arrow) can be seen in the space between the protoplasmic cylinder and cell envelope.* × *95,000.*

FIG. 3. *A spirochetal granule of strain FM containing several protoplasmic cylinders lacking cell envelopes. Segments of axial and terminal filaments (arrows) are seen within the granule.* × *162,000.*

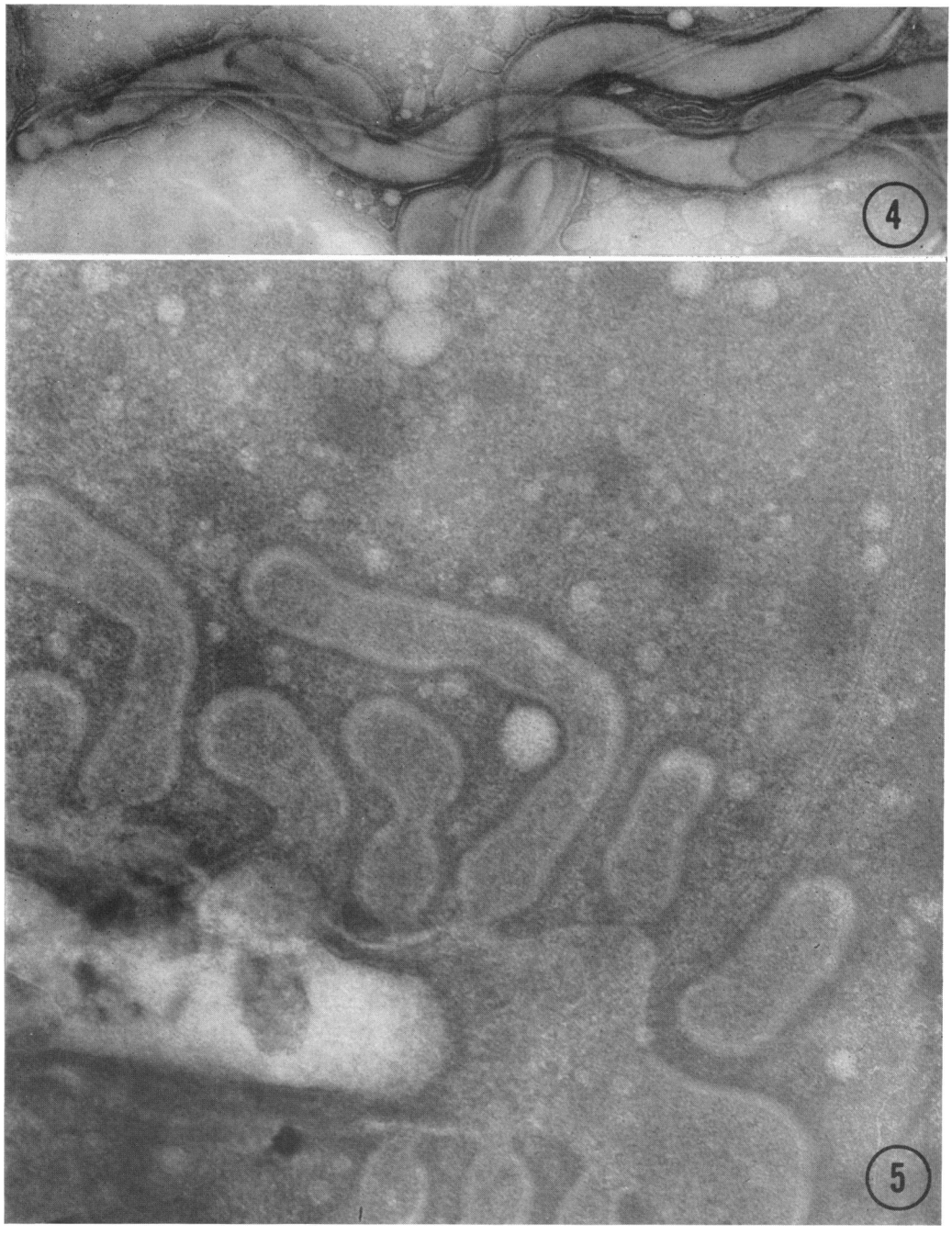


FIG. 4. *A negatively stained preparation demonstrating the bulbous tapered end of strain FM. The protoplasmic cylinder is straight at the end where the axial filament is loose.* × *51,000.*

FIG. 5. *The protoplasmic cylinder of strain N9 is covered by an amorphous-appearing layer having many rod-shaped projections constricted at their ends. Thin fibrils approximately 15 A wide originate at the protoplasmic cylinder and pass through the cell envelope into the surrounding area.* × *140,000.*

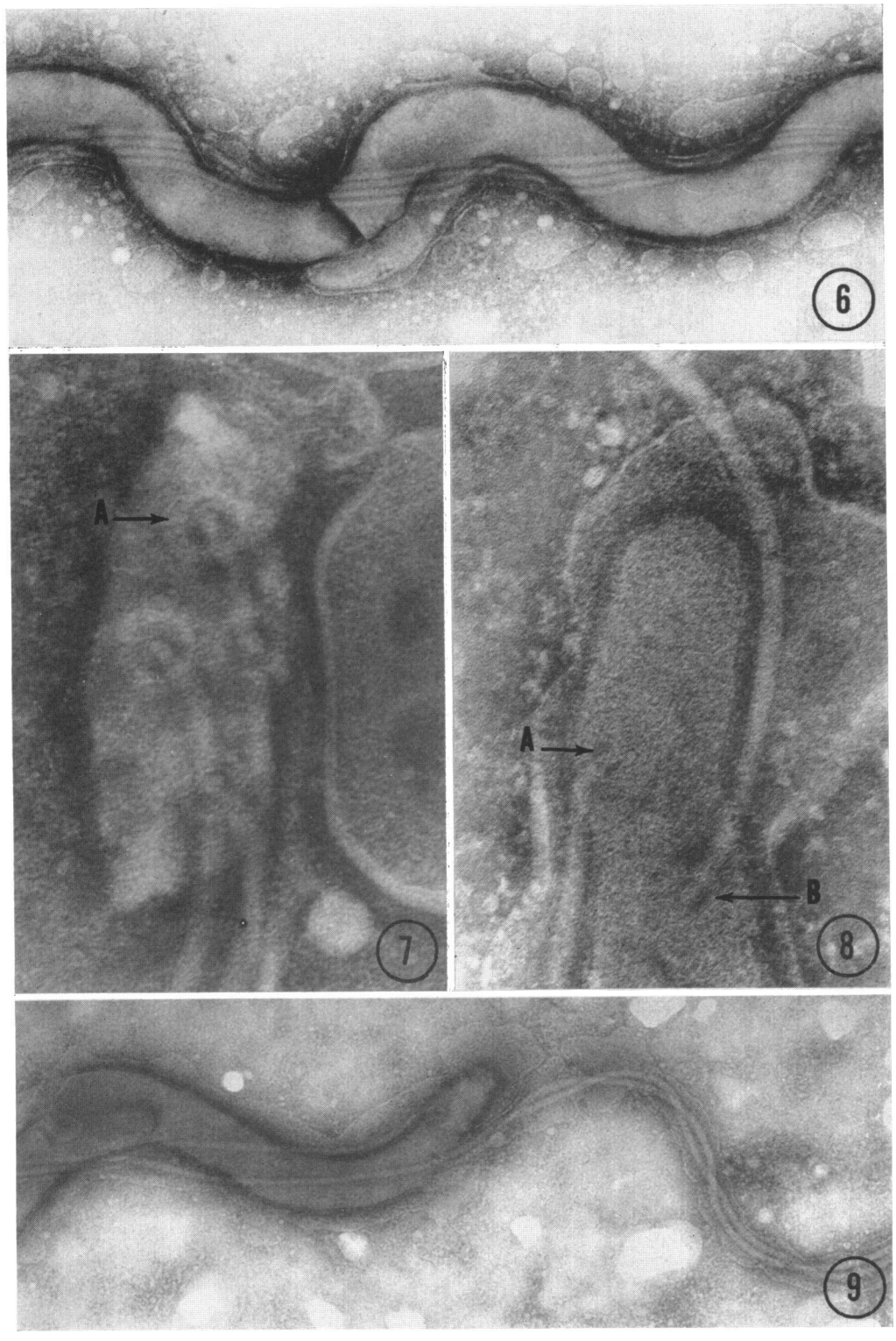


FIG. 6–9.

fiber of the axial filament of both FM and N9 either terminated in a small end knob approximately 0.3 to 0.4 $\mu$ from the end of the protoplasmic cylinder (Fig. 7 and 8) or continued beyond its end (Fig. 9). The end knobs were observed as dense circular structures (450 A) against a larger less-dense area (650 A). Occasionally, the outer circle had a morphology suggestive of subunits. Structures similar to end knobs were not seen except at the end of the protoplasmic cylinders.

Terminal filaments consisted of one to six separate fibers, approximately 200 A wide, which originated either by continuation of the axial filament through the cell envelope (Fig. 9) or from end knobs similar to, but not the same as, those of axial fibers (Fig. 8), or by both methods. Usually, the terminal filament possessed the same number of fibers as did the axial filament of a given spirochete. The fibers of the terminal filament were not in a fixed position with respect to each other and occasionally crossed over one another. The cell envelope of strain FM also acted as an envelope for the terminal filament (Fig. 10). In some areas, the terminal-filament envelope appeared to flatten out and had irregular edges, whereas in other areas it was immediately adjacent to the fiber (Fig. 11).

Occasionally, alternate dark and light bands with a periodicity of approximately 60 A were observed perpendicular to the long axis of the terminal filament of strain FM. The striated regions usually were seen in undistorted segments, and ranged from 170 to 700 m$\mu$ in length. They were not restricted to any special part of the filament, but could be found at any point along it. The periodicity transcended the width of the filament irrespective of the number of fibers present, and appeared as a structure of the envelope and not of the fibers (Fig. 12 and 13). The curvature of the striations suggested a tubular formation (Fig. 12). Occasionally, micrographs which indicated that the periodicity was in the fiber and not in the terminal-filament envelope were obtained (Fig. 14). Within the 60 A striations, there appeared to be a microperiodicity of approximately 20 A (Fig. 15).

A periodicity was observed in the terminal filament of N9 in only one instance. This filament did not have the discrete morphology usually apparent in the terminal filaments of N9, but rather an irregular periphery (Fig. 16). The cross-banding in this instance was approximately 75 A, slightly larger than that observed in strain FM.

Frequently, very thin fibrils which seemed to originate by a splitting of the fibers of the terminal filament of strain N9 were noticed. The fibrils measured approximately 15 A, and either were in single strands or formed large bundles (Fig. 17, arrow). In older cultures (7 days or more), many thin fibrils passing through the cell envelope were seen (Fig. 18). A similar splitting of the terminal-filament fibers was rarely observed in negatively stained preparations of strain FM.

## DISCUSSION

The observations of a triple-structured cell wall, as well as a concentric dense-light-dense arrangement within the protoplasmic cylinder, agreed with the findings of previous workers (Listgarten et al., 1963).

The cell envelope of spirochetes has been described both as a slime-like layer (Takeya, Mori, and Toda, 1957) and as an irregular envelope with a single electron-dense border approximately 140 A wide (Listgarten et al., 1963). In the present study, the cell envelope was comparable in position, but not in detailed structure to that previously reported. In all cases, the cell envelope appeared to have a triple-structured wall similar to the cell wall of the protoplasmic cylinder. The total width of the three layers was approximately one-half that given for the single dense border.

Occasionally, two or more protoplasmic cylinders devoid of individual cell envelopes were contained within a single limiting membrane

FIG. 6. *The spiral amplitude of strain FM is evident. Four fibers separated by a uniform space compose the axial filament.* × *65,000.*

FIG. 7. *Four separate fibers of the axial filament of strain N9 are attached to end knobs of the protoplasmic cylinder. The end knobs appear as a dense circular area approximately 450 A wide against a larger less-dense area of 650 A. A suggestion of substructure is evident at A.* × *180,000.*

FIG. 8. *One fiber of the axial filament of strain FM terminates in a small end knob (A); a separate fiber of the terminal filament begins in a similar end knob (B).* × *153,000.*

FIG. 9. *One fiber of the axial filament terminates at the end of the protoplasmic cylinder of strain FM; the other continues past the end and becomes part of the terminal filament.* × *53,000.*

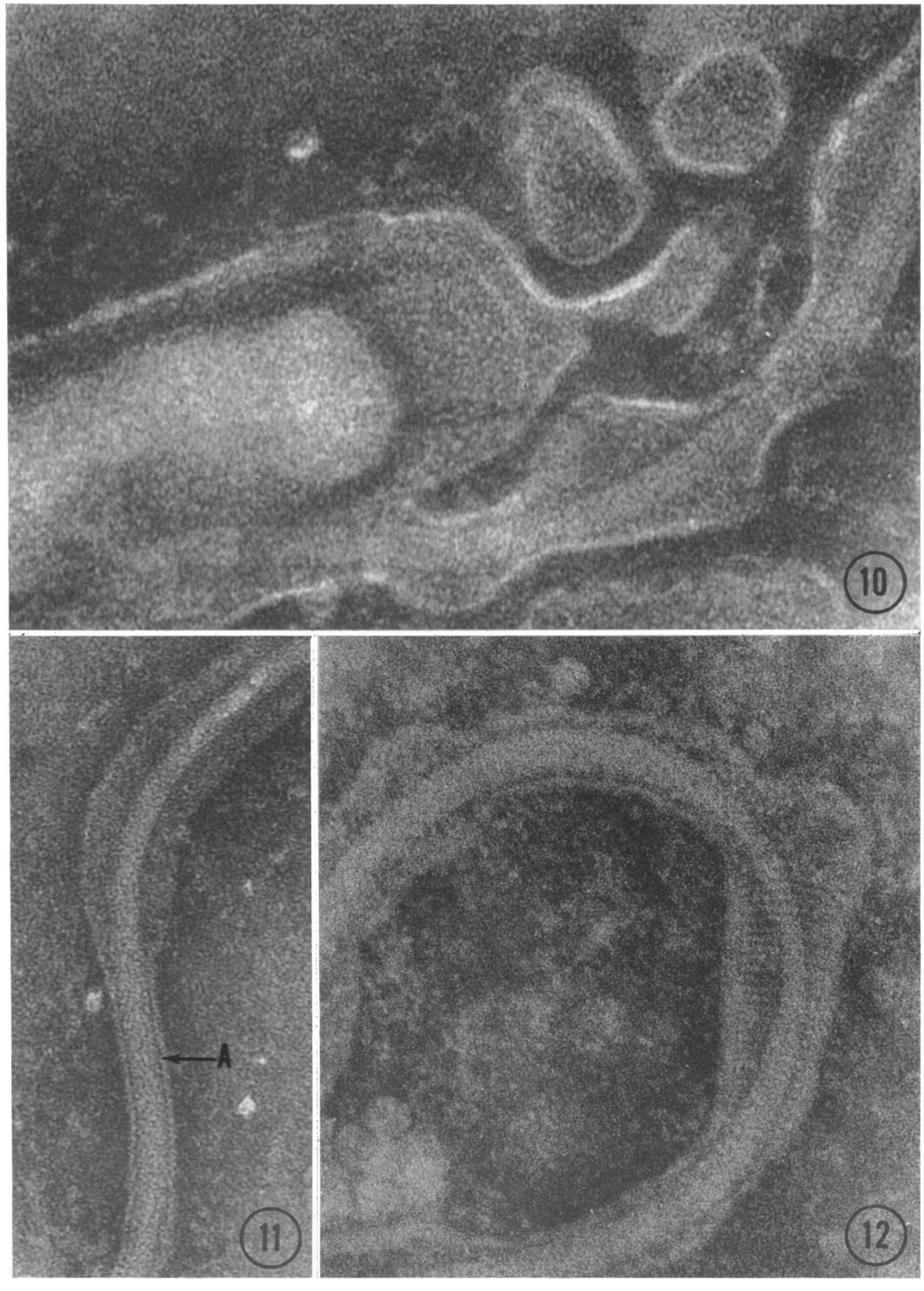


FIG. 10. *The cell envelope becomes the envelope of the terminal filament of strain FM.* × *180,000.*

FIG. 11. *The terminal-filament envelope of strain FM has flattened out in one area, and appears immediately adjacent to the fiber in another. The narrow dense line (A) indicates a deposit of phosphotungstic acid, and suggests that a space exists between the fiber and the envelope.* × *177,000.*

FIG. 12. *A 60-A periodicity transcends the width of the terminal-filament envelope of strain FM.* × *230,000.*

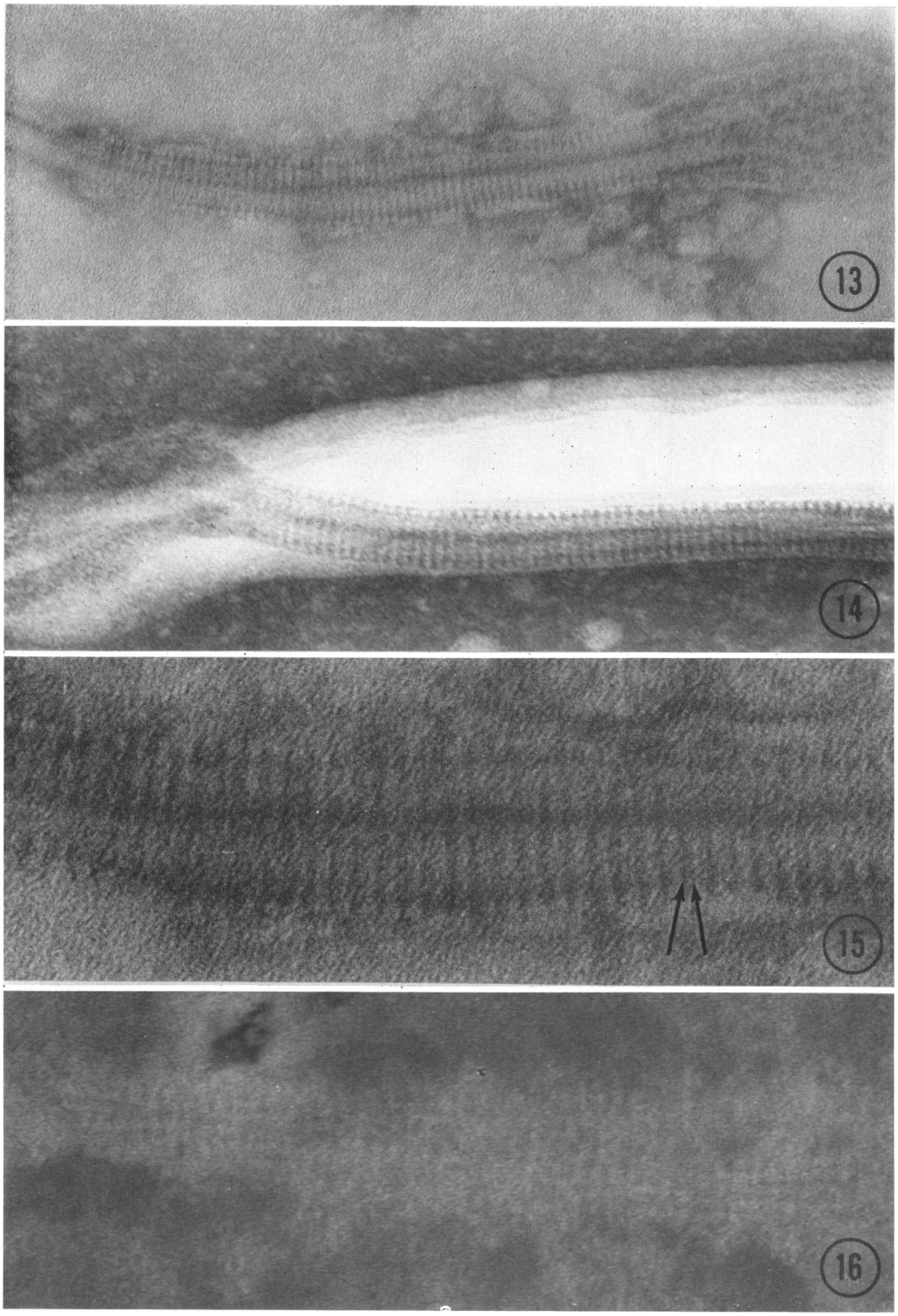


FIG. 13. *Another example of a 60-A periodicity in strain FM. The striations do not appear tubular as in Fig. 12, and are evident only where the filament envelope is present.* × *232,000.*

FIG. 14. *The 60-A striations appear to be in register between two fibers. The position of the terminal-filament envelope is not evident in this micrograph.* × *277,000.*

FIG. 15. *An enlarged area of Fig. 13 illustrating a subdivision of the striations into smaller units (arrow).* × *577,000.*

FIG. 16. *A 75-A periodicity is evident in the terminal filament of strain N9.* × *336,000.*

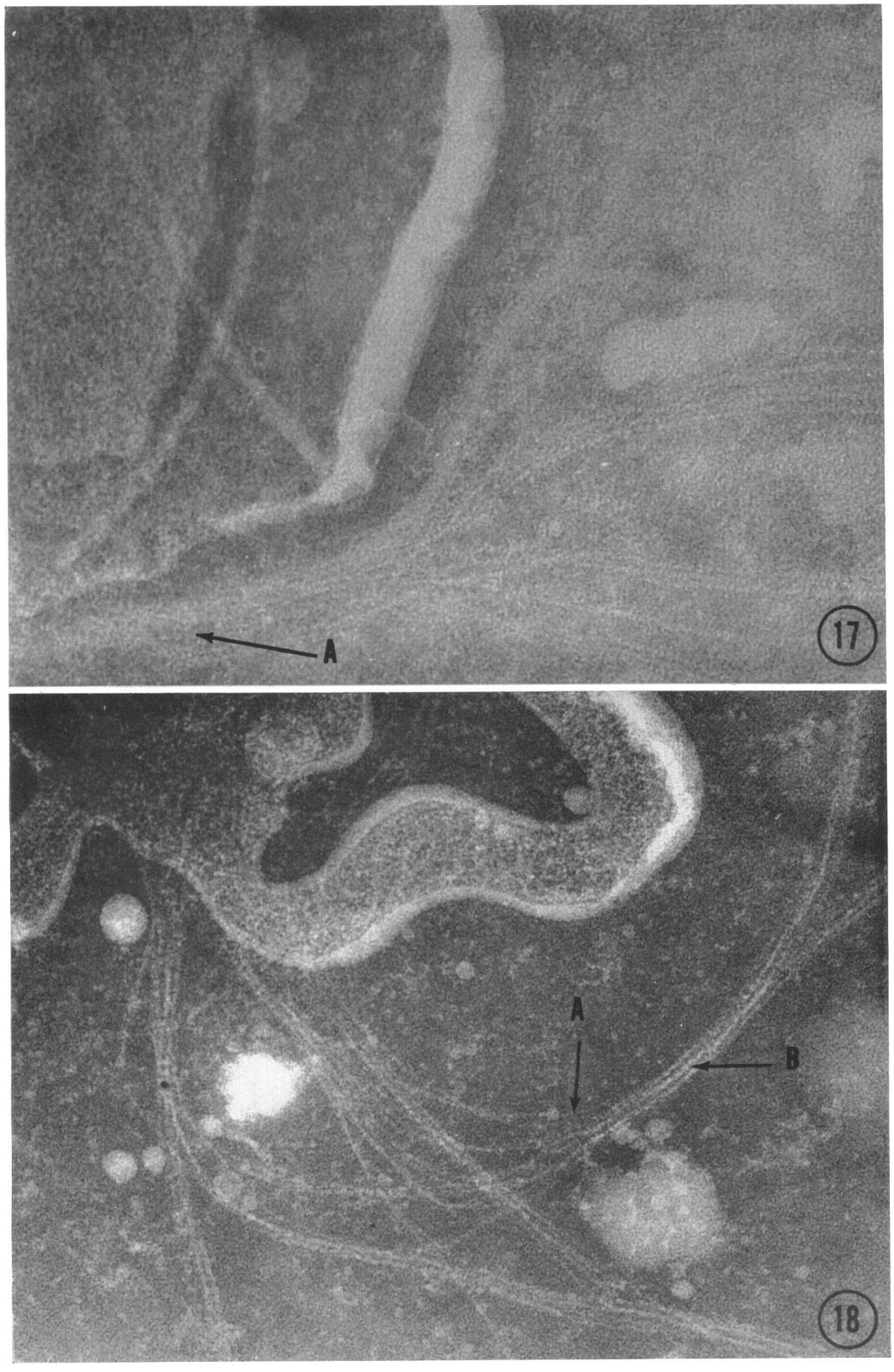


FIG. 17–18.

similar in morphology to the cell envelope, and the composite structure was representative of a spirochetal granule. The cell wall, cell envelope, and limiting membrane of the granules were generally comparable to the unit-type membrane seen as the walls of gram-negative bacteria (Kellenberger and Ryter, 1958; Robertson, 1959). The rod-shaped projections of the cell envelope of strain N9 were possibly artifacts, suggesting that the envelope was not comparable to a cell wall in rigidity, but rather to a cytoplasmic membrane which is considered less rigid.

Disagreement has prevailed concerning the location of the axial filament. Czekalowski and Eaves (1955) reported that the axistyle of *Leptospira* was outside the cell envelope, whereas Listgarten et al. (1963) demonstrated axial filaments in the space between the protoplasmic cylinder and the cell envelope. The work reported here concerning a *Treponema* and a *Borrelia* definitively illustrated that the axial filament, at least of these two strains, was situated inside the cell envelope.

Little variation has been reported in the number of fibers among organisms of most spirochetes. Swain (1955) reported that, in *Leptospira*, a single straight axial fiber was present, 3 fibers composed the axial filament of *T. pallidum*, and a bundle of up to 12 fibers was present in *Borrelia*. Listgarten et al. (1963) reported that *T. microdentium* had a pair of fibers wound around the length of the protoplasmic cylinder. In strains FM and N9, however, great variation existed. Individual organisms of FM exhibited from one to five fibers, whereas the fibers of N9 varied from one to six.

End knobs were usually evident on both ends of the organism and served as attachment sites for fibers of the axial and terminal filaments. Similar end knobs were reported in *L. pomona* by Czekalowski and Eaves (1955), who indicated that they anchored the axial filament to the protoplasmic cylinder. Swain (1955) demonstrated broken fibers attached to spirochetes which had straightened out, and suggested that the axial filament acted as a stiffener which maintained the spiral shape of the organism. Similar straight organisms were occasionally observed in this study. These end knobs may, therefore, serve two functions: maintenance of the necessary tautness of the axial filament to sustain the spiral character of the organism, and attachment for fibers of the terminal filament. It should be noted, however, that terminal-filament fibers were not attached to the same end knobs as were fibers of the axial filament. The end knobs were possibly analogous to the basal granules or blepharoplasts seen in flagellated bacteria, but this could not be determined from our results.

Early investigators reported the presence of "flagella" on a wide variety of spirochetes (Hampp et al., 1948; Morton, Rake, and Rose, 1951). Swain (1955) considered these flagella to represent fibers of the axial filament broken by washing the organisms in distilled water. The latter conclusion was supported by the present study of unwashed cultures of organisms identical to some of those previously studied by Hampp et al. (1948). No flagellar processes were seen, and the axial filament fibers usually remained in position. Occasionally, a broken fiber that protruded outward and resembled the flagellar appendages previously reported was observed.

The absence of readily identifiable flagella in the spirochetes posed the question of the mode of locomotion. Many authors suggested that the axial filament served this function as an undulating membrane (Babudieri and Bocciarelli, 1948). Others suggested that contraction and relaxation of the filaments must be considered as a possible mechanism of propelling the organism (Swain, 1955).

On this basis, therefore, it seemed logical to compare the filament structure to that of bacterial flagella, which belong structurally to the keratin, myosin, epidermin, and fibrinogen group of fibrous proteins (Burge, 1961). Two morphological arrangements were observed in this study in the terminal-filament envelope (FM) or the terminal filament (N9), which suggested a structural similarity to some proposed models of bacterial flagella: one, a splitting of the individual fibers of the terminal filament of strain N9 into fibrils approximately 15 A wide; the other, a periodicity of 60 A in the terminal-filament envelope of strain FM.

X-ray diffraction patterns of flagella from

FIG. 17. *Many 15-A fibrils can be seen to arise from a rather diffuse fiber (A), which was part of the terminal filament of strain N9.* × *160,000.*

FIG. 18. *A number of fibrils ranging in size from 15 A (A) to greater than 30 A (B) pass through the cell envelope of strain N9. Clusters of fibrils can be observed.* × *221,000.*

*Proteus vulgaris*, *Bacillus subtilis*, *Salmonella typhimurium*, as well as from other bacteria, were shown to be almost identical (Astbury, Beighton, and Weibull, 1955; Beighton, Porter, and Stocker, 1958). Certain of the equatorial reflections in these patterns were interpreted as being due to the packing of folded polypeptide chains (helices) within a flagellum (Astbury et al., 1955). Burge (1961) proposed two structural models for the bacterial flagellum based on the data of Astbury et al. (1955). In one model, a single flagellum was composed of three filaments (56 A), each consisting of 19 $\alpha$-helices; in the other, the flagellum contained seven filaments (30 A), each containing 7 $\alpha$-helices. The $\alpha$-helices were approximately 10 A wide.

The structural arrangement, especially in the N9 fibers, while contrasting in details such as number and size of subunits in a fiber, agreed in general with the fibrillar concept of flagellar composition. If we compare the electron microscopic data obtained here with the proposed models of Burge (1961), the 15 A fibrils would possibly represent $\alpha$-helices of polypeptide chains contained in the fibers. An $\alpha$-helix with a diameter of 15 A does not agree completely with the 10 A figure given by Burge (1961); this may be due to limitations of negative staining as well as inherent errors of measurement. No specific arrangement of fibrils in definite groups within the fiber, as suggested by Burge (1961), was evident. However, there was a tendency for several fibrils to cluster together. Further studies with X-ray diffraction techniques would be necessary to determine the spacing of individual fibrils within the fiber.

The 60 A spacing observed in the terminal-filament envelope of strain FM cannot be compared to the 410 A spacing observed in X-ray diffraction data of a flagellum (Astbury et al., 1955). No evidence of a 60 A spacing has been found in the X-ray diffraction patterns of bacterial flagella. Because most bacterial species studied do not possess an envelope comparable to the type observed in this study, and because the 60 A spacing appeared to be a part of the envelope itself, the absence of such a periodicity may be obvious.

Periodicity was observed only once in the terminal filament of strain N9, and was approximately 75 A. This was slightly larger than the 60 A banding observed with strain FM, a difference which could be explained by the somewhat degraded condition of the N9 filament or perhaps by species differences. The lack of a terminal-filament envelope in strain N9 in almost all the specimens could explain the rare occurrence of a similar periodicity as in strain FM. The significance of such a 60 A periodicity and its relationship, if any, to the motility of the organism is not, as yet, understood, and merits further study. It may represent, however, a transmission of a periodic contraction and expansion along the length of the filament, resulting in a whiplike motion.

Other models of flagella have been proposed. Kerridge, Horne, and Glauert (1962) examined isolated flagella of *S. typhimurium;* after partial degradation, spherical subunits corresponding to the flagellin molecule and having an estimated diameter of 45 A were observed and were arranged in accordance with hexagonal packing. Their observations do not agree with the X-ray diffraction studies of Burge (1961) on the flagella of *P. vulgaris*, nor do they concur with the electron microscopic data presented here. The different results of studies pertaining to flagella (or flagella-like appendages) only serve to illustrate that probably no single architectural arrangement will be found in all organs thought to be associated with movement.

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